

***In vivo* Antagonistic Effect of *Lactobacillus helveticus* CU 631 against *Salmonella enteritidis* KU101 infection**

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ABSTRACT : *In vivo* antagonistic effect of *Lactobacillus helveticus* CU 631 and *Lactobacillus* spp. against typical enteritis causing pathogen *Salmonella enteritidis* KU 101 have been determined, which showed an increase in survival rate and the decline in viable cell numbers of pathogen in liver and spleen at sacrifice. A significant difference in the antagonistic effect against KU 101 were observed, which was species and/or strain dependent of *Lactobacillus* ($p < 0.01$), the survival rate of the mice in the *Salmonella* infection by feeding *L. helveticus* CU 631 has been shown to be 157%, whereas those of *L. rhamnosus* GG ATCC 53103, *L. acidophilus* ATCC 4356, *L. johnsonii* C-4 were 137%, 132%, 119% respectively on the basis of lactobacilli non-associated control KU101 fed mice to be 100%. Viable cells of *S. enteritidis* KU101 in the liver and in the spleen at sacrifice were decreased in *Lactobacillus* spp. fed group with no significant difference. The higher level of total secretory IgA concentration in the intestinal fluid of lactobacilli fed mice than control mice have been observed. *In vitro* antagonistic activity of *Lactobacillus* spp. against KU101 have been determined, a prominent antagonistic activity of CU 631 against KU 101 were demonstrated. (*Asian-Aust. J. Anim. Sci.* 2003. Vol 16, No. 3 : 430-434)

Key Words : Antagonistic Effect, *Lactobacillus* spp. IgA, *Salmonella enteritidis*

INTRODUCTION

Many strains of *Salmonella enteritidis*, *E. coli* and *Campylobacter* are reported to be agents of enteritis and food intoxication with principal symptoms of diarrhea. They inhabit in the gastrointestinal tract of domestic animals and are transmitted to animal and food products, hence they are recognized as an important hygienic indicator organism which causing acute enteritis and septicemia (Aserkoff et al., 1970; Bowmer, 1964).

Diarrheal disease has been one of the critical diseases and it can be fatal when it occurs in individuals with compromised immune system including infants and the elderly (Black, 1993). The control of these bacteria are of pivotal importance in preventing diarrheal diseases in humans and animals (Anon, 1990), The gastrointestinal tract contains a complex of microorganisms whose population's change are susceptible in response to various kinds of environmental factors such as antibiotics, medication, and diet. Enterobacterial pathogens may induce a disease when the host's normal flora has been disturbed, as diarrhea usually results from the disruption of the normal flora in the gastrointestinal tract (GI), the treatments that recovering the normal flora have been to be effective with the least side effects. In this respect, probiotics that have potentials to improve the microbial balance in the GI tract have long been used for the prophylactic and therapeutic agents against diarrheal diseases (Kateralis et al., 1995;

Gonzales et al., 1994). The lactic acid-secreting bacteria such as lactobacilli and bifidobacteria work as probiotics (Gibson and Fuller, 2000). However not all lactic acid bacteria can be probiotic and their potencies can be different depending on strains, and good strains should be developed and characterized for a practical use.

In search for the probiotics exhibiting preventative effects against diarrheal diseases we evaluated 4 species of *Lactobacillus* with the *Salmonella* infection model in mice. *Salmonella* is the second most common cause of diarrheal diseases in the United States (Edwards, 1999). The antagonistic effect of *Lactobacillus* spp. against *Salmonella enteritidis* KU101 were determined.

MATERIALS AND METHODS

Mice

Male specific pathogen free ICR mice were obtained from Daehan Biolink (Choongbook, Korea) and were acclimated for at least 1 wk before use. The mice were kept under the condition of 12 h light-dark cycle at a controlled temperature ($22 \pm 2^\circ\text{C}$) and were supplied with water and feed *ad libitum*.

Bacterial strains and media

The strain and sources of bacteria used in this study were given in Table 1. *Lactobacillus* spp. were cultured in MRS broth (Difco, USA) at 37°C and maintained in 11% skim milk containing 0.75M adonitol at -70°C . *Salmonella enteritidis* was grown in Brain Heart Infusion (Difco, USA) and maintained in BHI broth containing 30% glycerol at -70°C .

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Table 1. Sources of strains of *Lactobacillus* spp., *Salmonella enteritidis* used in this investigation

	Strains	Source
<i>L. helveticus</i>	CU 631	Dairy Microbiol. Lab. Chung- Ang Univ.
<i>L. johnsonii</i>	C - 4	Food Research and Development Center of Canada
<i>L. rhamnosus</i> GG	ATCC 53103	"
<i>L. acidophilus</i>	ATCC 4356	"
<i>Sal. Enteritidis</i>	KU 101	Veterinary Microbiol. Lab. Kunkuk Univ.

***In vivo* antagonistic effect test against the *Salmonella* infection in mice**

The lactobacilli were subcultured in 10 ml of MRS broth (Difco Laboratories) at 37°C overnight, the whole grown cells were transferred to 50 ml of same broth and were further incubated at 37°C, when the cell growth reached the log phase, the cells were harvested by centrifugation and were suspended in 10% skim milk with a desired suspension. The bacteria a dose of 2.4×10^9 cfu were administered orally to the mice for 8 consecutive days, the mice in the control group received skim milk only. One day after the last feeding was done, ICR mice (five per group) were orally challenged with 1×10^8 cfu of *S. enteritidis*, the dose of which is equivalent to LD₅₀. And the percent survival (number of alive/total number of mice) was recorded every day for 21 days.

Viable cell count of *S. enteritidis* in liver and spleen

Viable cell count was conducted on the method described by Perdigon (1992). Liver and spleen tissue were mixed in 5 ml of 0.1% peptone and homogenized by homogenizer. *Salmonella-Shigella* agar (Difco, USA) was used and followed by the procedures of standard plate count (Marshall, 1993). Diluted samples were plated on SS agar and incubated for 24 h at 37°C and black colonies were counted

In vitro* antagonistic activity of *Lactobacillus* spp. against *Salmonella enteritidis

The ultrasensitive assay of Lehrer et al. (1991) was used to determine the antimicrobial activities of *Lactobacillus* spp. MRS culture against *Salmonella enteritidis* KU101 which were grown overnight for 18 h at 37°C in 50 ml of tryptic soy broth (TSB), to obtain midlogarithmic phase KU101, 10 ml of tryptic soy broth was inoculated with 200 µl of cultured tryptic soy broth overnight culture and incubated additional 3 h at 37°C. The bacteria were pelleted by centrifugation at $5,500 \times g$ for 5 min at 4°C, washed once with PBS and resuspended in 10ml of cold PBS. The optical density (OD) of an aliquot was measured at 620nm and based on the relationship $OD_{620} 0.2 = 5 \times 10^7$ CFU/ml, a

volume containing 1.5×10^6 CFU was added to 10 ml of previously autoclaved, warm (42°C) 10 mM sodium phosphate buffer containing 3 mg of powdered TSB medium, 1%(w/v) of low-electroendosmosis-type agarose (Sigma no 6013, St Louis Mo), and a final concentration of 0.02% (v/v) Tween 20 (Sigma). After rapid dispersal with a laboratory vortex mixer, the agar was poured into petri dish to form a uniform layer approximately 1 mm deep. A 3 mm diameter gel punch was used to make nine evenly spaced wells per dish, *Lactobacillus* spp. samples (5 µl) were added to each well. The plates were incubated for 3 h at 37°C and overlaid with 10 ml of sterile agar (TSB[6%, wt/vol], agarose [1% wt/vol]). After incubation for 18 to 24 h at 37°C, the diameter of the inhibition area surrounding the wells was measured. The diameter of clearing was calculated by subtracting the diameter of central well (3 mm) and expressed in units (0.1 mm=1 U).

Determination of the level of sIgA from the intestinal contents

The intestinal contents from the ICR mice fed with *Lactobacillus* spp. were prepared; the inside of the small intestine was flushed with 1 ml of ice-cold PBS and centrifuged $16,000 \times g$ for 1 min and supernatant was taken in 1 ml PBS containing 0.1 mg/ml soy bean trypsin inhibitor, 50 mM EDTA (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma), and the concentration of s Ig A from resulting intestinal contents were determined by the procedures described by Han et al. (1999).

50 µl of 0.1% bovine serum albumin (Sigma, USA) was added into the each wells of 96 well microplate (Falcon, USA), 50 µl of sample and 50 µl of intestinal content was added to the first well and mixed, the diluted samples were incubated 2 h at 37°C and maintained overnight at 4°C. And removed the samples from the plate and washed 3 times with cold PBS containing 0.05% Tween 20.

200 µl of bovine serum albumin was added to each well and incubated 1 h at 37°C and washed 3 times with cold PBS (pH 7.4) containing 0.05% Tween 20. 50 µl of anti-mouse IgA (Sigma USA) conjugated with horse sera peroxidase which was diluted by 1:1,000 was added to each well and incubated 1 h at 37°C and washed 3 times with cold PBS (pH 7.4) containing 0.05% Tween 20.

100 µl of alkaline phosphatase substrate solution containing 1 mg/1 ml of p -nitrophenyl phosphate disodium (Sigma, USA) was added to each well and incubated 2 h at 37°C and determined the total IgA by measuring the optical density at 405 nm with ELIZA reader (Bio-rad Microplate reader Model 3550, USA).

Statistical analysis

Within the same treatment group, for the comparison of

the inhibitory activity by *Lactobacilli*, *in vivo* protection activities between the treatments values were compared using SAS Duncan's multiple-range test.

RESULTS AND DISCUSSION

***In vivo* antagonistic effects of *Lactobacillus* spp against the *Salmonella* infection**

To investigate if the CU 631, ATCC 4356, ATCC 53103 and C-4 monoassociated bacteria are able to protect the specific germfree mice against *Salmonella enteritidis* KU101 infection, and to screen the inhibitory strain that have preventive capacity against diarrheal disease, mice were infected with *S. enteritidis* KU101. The ICR mouse were fed with each *Lactobacillus* for 8 consecutive days, one day after last feeding *S. enteritidis* were challenged orally, when the survival rates were observed every day for 21 days. The mice started to become sick and the first death was observed on day 3 postinfection and a 32% mortality

rate was observed by day 11 postinfection KU101 infected control mice, but not until 21 postinfection in the CU 631 monoassociated KU101 infected mice. As shown Figure 1(A), a highly delayed mortality was observed in the CU 631 monoassociated KU101 infected mice compared with the germ free KU101 infected mice ($p < 0.01$) The most active in mortality delaying activity have been observed in *L. helveticus* CU 631, and followed by *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 53103, and *L. johnsonii* C-4, with the *Salmonella enteritidis* KU 101 infection model in mice. The results indicate that the established lactobacilli species exert antimicrobial activity *in vivo*.

Figure 1(B) showed a strong effect of CU 631 on survival rate of mice (survival rate of 157%) comparing with the lactobacilli non-associated control mice infected with KU 101. Those of ATCC 4356, C-4 and ATCC 53103 monoassociated in the intestine of KU 101 infected mice revealed 137%, 132% and 119% respectively. In review report, Marie-Francoise et al. (1997) concluded that the

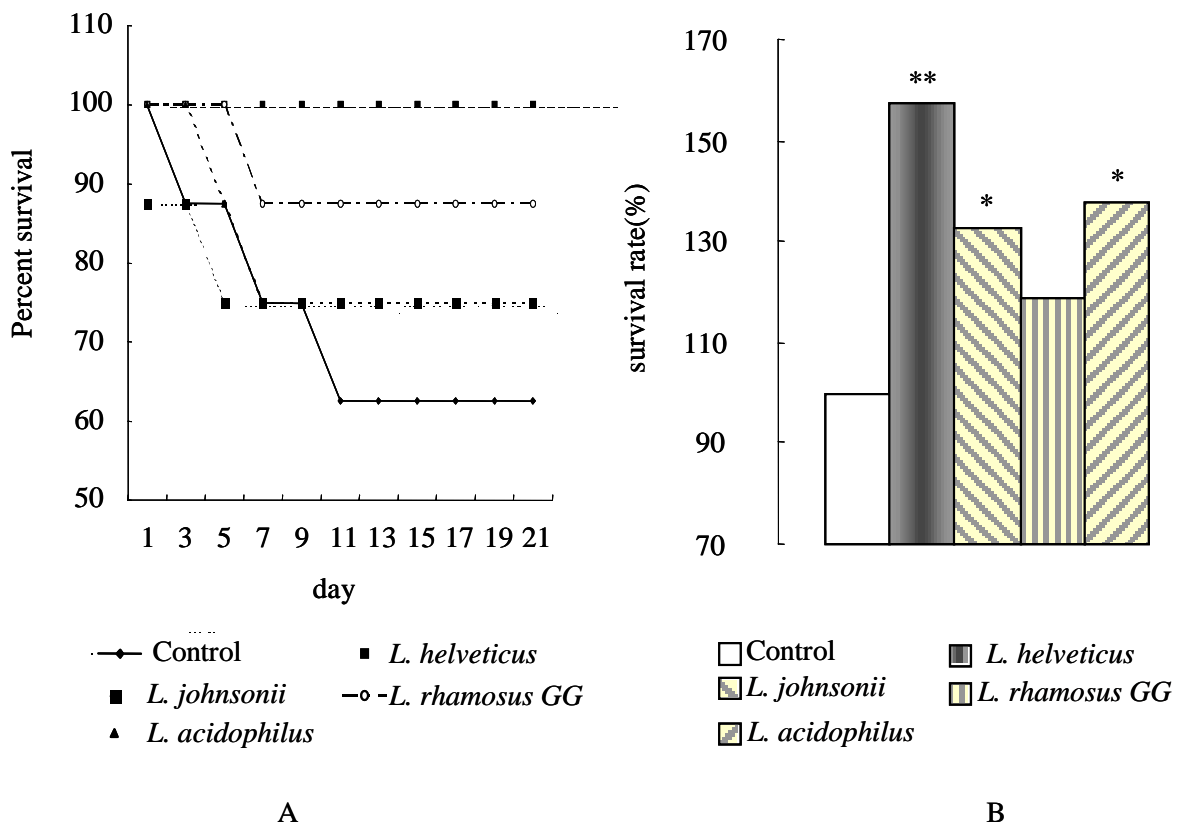


Figure 1. (A) Mortality due to *S. enteritidis* KU101 infection in germ free ICR mice associated with *Lactobacillus* spp. CU 631, ATCC 4356, ATCC 53103 and C-4 and not associated(control)mice. Mice were fed with *Lactobacillus* spp., following challenge with *Salmonella enteritidis* 1×10^8 cfu (B) Survival rate of mice fed with *Lactobacillus* spp. following challenge with *Salmonella enteritidis* after 21 days.

$$\text{Survival rate (\%)} = \frac{\text{Mean survival times (hours) of treated mice}}{\text{Mean survival times (hours) of control mice}} \times 100$$

*, a-d Means in a row with no common superscript differ significantly ($p < 0.001$). SEM (Standard error of mean):1.30.

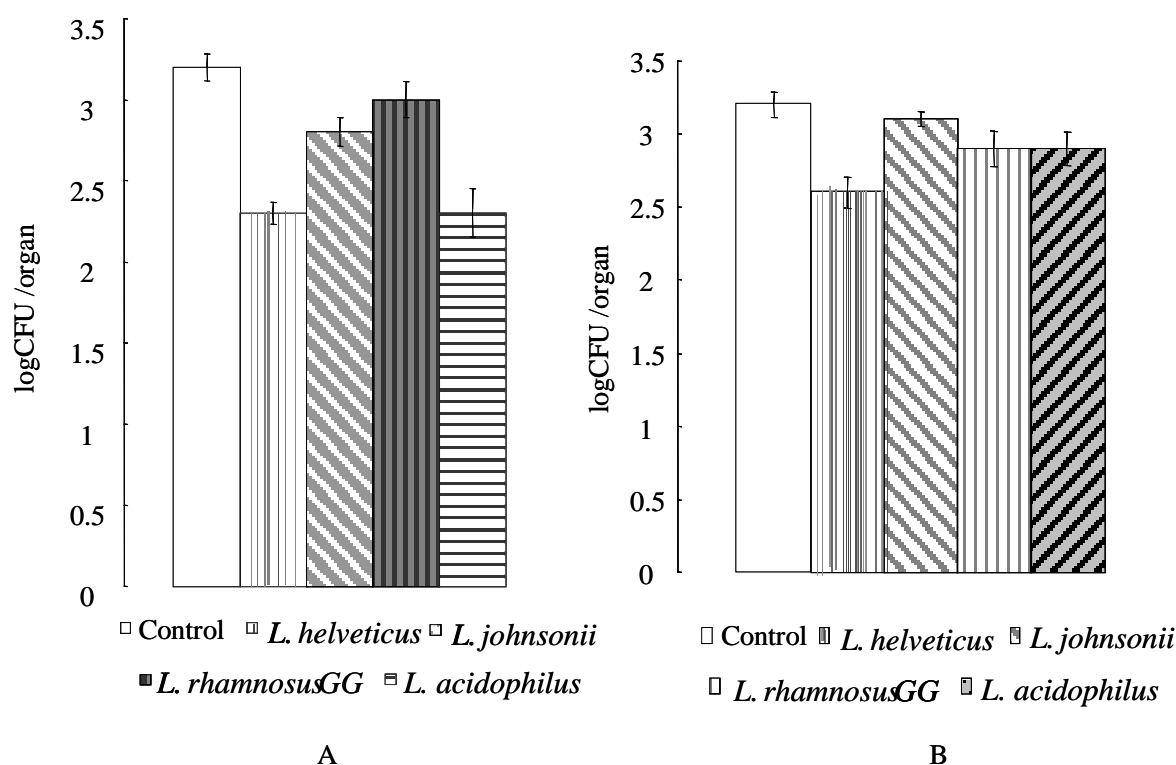


Figure 2. The number of *Salmonella enteritidis* in the (A) liver and (B) spleen of the mice fed with *Lactobacillus* spp., 21 days after the challenge with *Salmonella enteritidis*

adhering human *Lactobacillus acidophilus* strain LA1 demonstrated an antibacterial activity in conventional or germ free mouse models orally infected by *Salmonella typhimurium*. *L. casei* GG survives in the human gastrointestinal tract and causes clinically significant health benefits against human diarrhea (Isorauri et al., 1994).

In vivo antagonistic effect of CU 631 and lactobacilli have been shown in liver. (A) and in spleen (B) in Figure 2. The number of *Salmonella enteritidis* KU101 in the liver of the control mice was 3.2 ± 0.08 CFU log/organ but those numbers in CU631 fed mice were 2.3 ± 0.07 CFU log/organ with no significant difference. KU101 viable count in the spleen of the mice fed with *Lactobacillus* spp. on 21 days after the challenge was 2.6 ± 0.10 CFU log/organ, which

could be considered as the result of the protective and antagonistic activity of lactobacilli *in vivo*. There was no statistically significant differences in viable number of KU 101 in the *lactobacilli* treated mice as shown in Figure 2(B).

***In vitro* inhibition of *S. enteritidis* by *Lactobacillus* spp**

In vitro inhibition of *S. enteritidis* by *Lactobacillus* spp were given in Table 2. *L. helveticus* CU 631 showed most potent inhibition activity against *Salmonella enteritidis*, which agrees with those results of *in vivo* antagonistic effect test. Those results of *in vitro* and *in vivo* antagonistic effect test shows the fact that *Lactobacillus helveticus* CU 631 could be utilized as a probiotic starter strain. Lactobacilli are the most frequently used species in products for human consumption and can be found in infant foods, cultured milks and pharmaceutical preparations. *L. rhamnosus* GG survives in human gastrointestinal tract and causes clinically significant health benefits against human diarrhea (Isolauri et al., 1994; Kaila et al., 1992). In this research, a statistically significant inhibitory activity of *L. helveticus* CU 631 against *S. enteritidis* than that of *L. rhamnosus* GG has been shown in Table 2, *in vitro* studies have recently documented the antagonistic activity of 27 lactobacilli strains against enteropathogen *Helicobacter pylori* as a result of the competitive exclusion of adhesion of pathogenic bacteria to host cells CU 631 revealed most

Table 2. *In vitro* antagonistic effect of *Lactobacillus* spp. against *Salmonella enteritidis*

Species	Clear zone diam (U)
<i>L. helveticus</i> CU 631	9.50 ^a
<i>L. johnsonii</i> C-4	8.00 ^b
<i>L. rhamnosus</i> GG ATCC 53103	8.50 ^b
<i>L. acidophilus</i> ATCC 4356	6.00 ^c
SEM	0.18

1 unit = 1 mm.

^{a-d} Means in a row with no common superscript differ significantly ($p < 0.001$).

SEM: Standard error of mean.

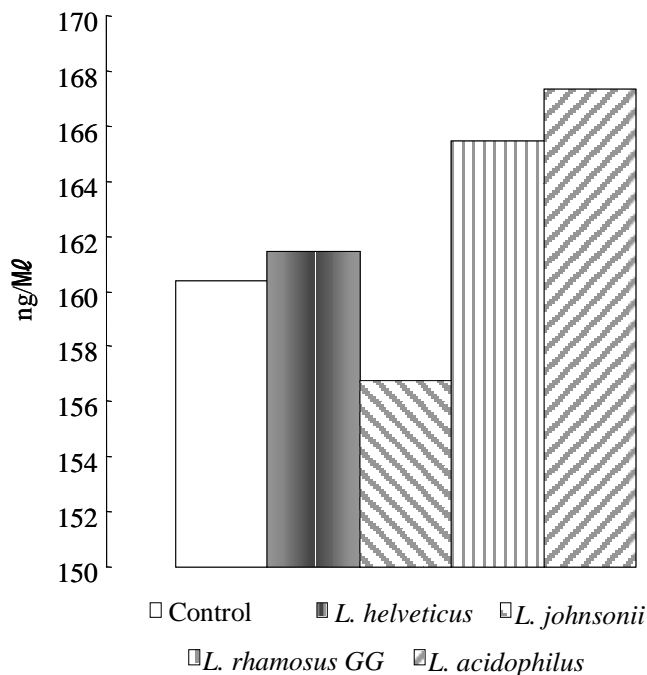


Figure 3. Effect of *Lactobacillus* spp. feeding on total secretory IgA production in small intestinal fluid, 21 days after the challenge with *Salmonella enteritidis*

potent antagonistic activity (Yoon and Won, 2002).

Effect of *Lactobacillus* spp. feeding on total secretory IgA production

Previous studies have shown that some probiotics administered orally exhibit their beneficial effects by stimulating gut-associated lymphoid tissue (GALT) (Perdigon et al., 1992). As shown in Figure 3, while the concentration of IgA in the *L. helveticus* fed group was an average of 162 ng/ml, that of control group remained an average of 160 ng/ml, indicating that there is no significant change in the total IgA level by feeding *L. helveticus* cells. *L. acidophilus* ATCC 4356 fed group showed the highest IgA level in the intestinal fluid among the *Lactobacillus* spp. fed group. In parallel with the antimicrobial activity, lactobacilli are known to stimulate immunological defences against pathogens (Perdigon et al., 1992), it has also been demonstrated that when *L. acidophilus* was orally administered to human in fermented milk, it was able to increase the blood phagocytic activity, as well as both the total immunoglobulin A levels in serum and the specific immunoglobulin A titers to *S. typhimurium* TY 21a in serum (Link-Amster et al., 1994), but our results indicated that the total Ig A level have been varied depending upon the lactobacilli strains as shown in Figure 3. Highly increased IgA level have been appeared in the *B. longum* fed mice; the concentration of secretory IgA in intestinal content from *B. longum* treated group was 2.5 times higher

than that of control group suggesting that the protective effects of *B. longum* may partly be attributed to the enhancement of local rather than systemic immunity (Han et al., 1999).

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